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Evaluation of a generic RT-nested-PCR for detection of flaviviruses in suspected fatal cases of dengue infection, Rio de Janeiro, Brazil

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A B S T R A C T

Flaviviruses are significant causes of disease worldwide and can be classified serologically into several antigenic complexes. The purpose of the present study was to evaluate the effectiveness of a generic RT-nested-PCR for detection of flavivirus during a dengue outbreak in Brazil in 2008. A total of 105 serum samples were collected from patients with fatal outcome and examined by generic RT-PCR, conventional RT-PCR, and IgM serology. The generic RT-PCR confirmed 19 of 105 (18%) cases. Conventional RT-PCR performed on 105 serum samples detected 45 (42.8%) dengue virus infections. The IgM serology confirmed 44 of 102 (43.1%) cases. The infecting serotype was identified by generic RT-PCR in 19 cases (18 DENV-2 and 1 DENV-3) and by conventional RT-PCR in 45 cases (40 DENV-2 and 5 DENV-3). In addition, we analyzed the performance of the generic and conventional RT-PCRs and IgM serology on serum samples stratified by the day of onset of symptoms. Our results indicate that different methods should be included in flavivirus surveillance programs, including virological and serological approaches.

1. Introduction

The genus *Flavivirus* is characterized by a single-stranded plus-sense RNA genome of approximately 11 kb, constituted by a single open reading frame (ORF) flanked by an untranslated region (UTR) in the 5′ and 3′ termini (Lanciotti et al., 2000). The ORF codes for three structural proteins are capsid (C), membrane (prM/M), and envelope (E), while those for seven non-structural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990).

The genus *Flavivirus* includes more than 70 arthropod-borne viruses that can cause severe encephalitis, hemorrhagic fever, and febrile illness in humans (Monath et al., 1996). Mosquito-borne flaviviruses represent a serious public health issue in Brazil, with Dengue viruses (DENV), Saint Louis Encephalitis virus (SLEV), Bussuquara virus, Cacipacore virus, Iguape virus, Ilheus virus, Rocio virus and Yellow Fever virus (YFV) being isolated from mosquitoes, animals, or humans (Figueiredo, 2000).

In South America, the West Nile virus (WNV) has been reported in neighboring countries of Brazil, including Venezuela (Bosch et al., 2007), Colombia (Berrocal et al., 2006), and Argentina (Morales et al., 2006). For these reasons, the implementation of virological methods may play an important role for rapid diagnosis of new flaviviruses in the country. The purpose of the present study was to evaluate the effectiveness of a generic RT-nested-PCR for detection of flavivirus during a dengue outbreak in Brazil in 2008.

2. Materials and methods

2.1. Patients and samples

The 105 suspected dengue fatal cases included in this study had acute febrile illness with two or more of the following clinical symptoms: headache, retrobulbar pain, myalgia, arthralgia, rash, and hemorrhage. All samples were received refrigerated and separately from private and public hospitals in the metropolitan area of Rio de Janeiro city and stored at −70 °C until tested. All samples were collected between January and May 2008 from patients ranging in age from 5 days to 90 years old. Ethical clearance was obtained with the approval resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil.

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[1] In memoriam.

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2.2. RNA extraction

Viral RNA for the conventional RT-PCR and generic RT-PCR assays was extracted from 140 μL serum samples by the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA), in accordance with the manufacturer's suggested protocol. RNA was eluted in 60 μL of buffer AVE and stored at −70 °C.

2.3. Conventional reverse transcriptase PCR assay

The conventional RT-PCR protocol for DENV detection and typing was performed on 105 serum samples, as described previously by Lanciotti et al. (1992). Briefly, cDNA copies of a portion of the viral genome (capsid/prM) were produced and amplified using two consensus primers designed to anneal to any of the four dengue virus types. Second-round amplification with type-specific primers results in different bands of DNA, characteristic of each dengue virus type, which can be observed directly in 1% agarose gel stained with ethidium bromide. Positive and negative controls were included in all steps.

2.4. Generic reverse transcriptase PCR assay

The generic RT-PCR protocol for *Flavivirus* detection was performed on 105 serum samples, as described previously by Sánchez-Seco et al. (2005). Degenerated primers were designed based on conserved motifs in a region of gene NS5. Positive controls tested by this method were DENV-1, DENV-2, and DENV-3 (obtained from Flavivirus Laboratory, Fiocruz, Brazil), and YFV (17D) (obtained from Biomanguinhos, Fiocruz, Brazil).

2.5. Nucleotide sequencing and phylogenetic analyses

Products from the second round of generic amplification were purified using the PCR purification kit or gel extraction kit (Qiagen, US). Sequencing reactions on both strands were performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, US), and analyzed using an ABI Prism 3730 Sequencer (Applied Biosystems, US).

Electropherograms were visualized by Chromas software version 1.45 (Technelysium Pty. Ltd., Queensland, Australia). Nucleotide sequences were aligned and analyzed using the Clustal X program (Thompson et al., 1997) and later edited by hand. All alignments are available from the authors upon request.

Representative sequences from 10 flaviviruses and arboviruses were retrieved from Genbank (www.ncbi.nlm.nih.gov), including DENV1-4, SLEV, YFV, WNV, Tick-borne encephalitis virus, Powassan virus, and Japanese encephalitis virus. A phylogenetic tree was constructed using MEGA 4 software (Tamura et al., 2007), using the Neighbor Joining (NJ) method and the Tamura Nei model. A bootstrap of 1000 replications was used to estimate the reliability of the predicted tree.

2.6. Serological test

Dengue IgM-capture enzyme-linked immunosorbent assay (ELISA) (PanBio, Brisbane, Australia) was performed on 102 serum samples, according to the manufacturer's instructions.

3. Results

A total of 105 serum samples were collected from patients with fatal outcome and examined by generic RT-PCR, conventional RT-PCR, and IgM serology to evaluate the effectiveness of the generic RT-PCR for use as a diagnostic tool for flaviviruses. Dengue infection was confirmed in 60% (63/105) of cases by the combined

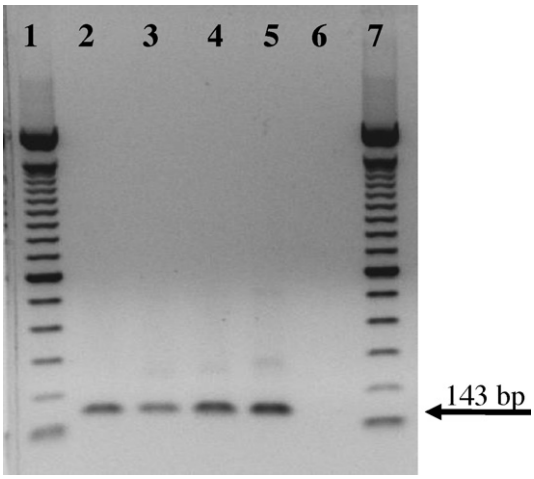

Fig. 1. Agarose gel electrophoresis of generic RT-PCR amplicons. Line 1, 100-bp DNA ladder; lines 2–5, positive controls; line 2, DENV-1; line 3, DENV-2; line 4, DENV-3; line 5, YFV (17D); line 6, negative control; line 7, 100-bp DNA ladder.

results obtained by generic RT-PCR, conventional RT-PCR, and IgM serology. The generic RT-PCR confirmed 19 of 105 (18%) cases. All positive controls tested by generic RT-PCR (DENV1-3 and YFV) rendered positive amplification (Fig. 1). Conventional RT-PCR performed on 105 serum samples detected 45 (42.8%) DENV infections. From those 45 cases positive by RT-PCR, 8 (17.7%) were positive only by this method. The IgM serology confirmed 44 of 102 (43.1%) cases. From those 44 cases positive by IgM serology, 18 (40.9%) were positive only by this method.

The infecting serotype was identified by generic RT-PCR in 19 cases (18 DENV-2 and 1 DENV-3) and by conventional RT-PCR in 45 cases (40 DENV-2 and 5 DENV-3). All 19 cases positive by generic RT-PCR were confirmed by the conventional RT-PCR. The performance of the generic RT-PCR and conventional RT-PCR (RT-PCRs) and IgM serology in serum samples, stratified by the day of onset of symptoms, is shown in Fig. 2. As expected, the molecular methods were more efficient until the fifth day of illness, and IgM serology was more efficient after the eighth day of illness (Fig. 2).

Double-stranded DNA products amplified in the generic nested PCR were directly sequenced as described in Section 2.5. The dengue sequences obtained in this study were submitted to GenBank (accession nos. FJ390025, FJ390026, FJ390027, FJ392583, FJ392584, FJ392585, FJ392586, FJ392587, FJ392588, FJ392589, FJ392590, FJ392591, FJ392592, FJ392593, FJ392594, FJ392595, FJ392596, FJ392597, and FJ392598). DENV-2 and DENV-3 were identified by phylogenetic analysis (Fig. 3), confirming the results obtained by conventional RT-PCR.

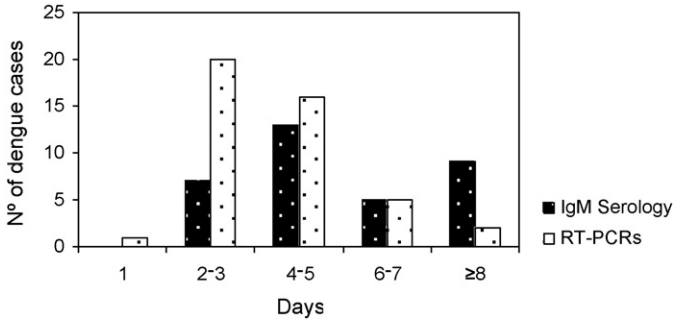

Fig. 2. Detection of dengue virus by day of illness by IgM serology and molecular methods (conventional RT-PCR and generic RT-PCR) in fatal cases during an outbreak in Rio de Janeiro, Brazil, 2008.

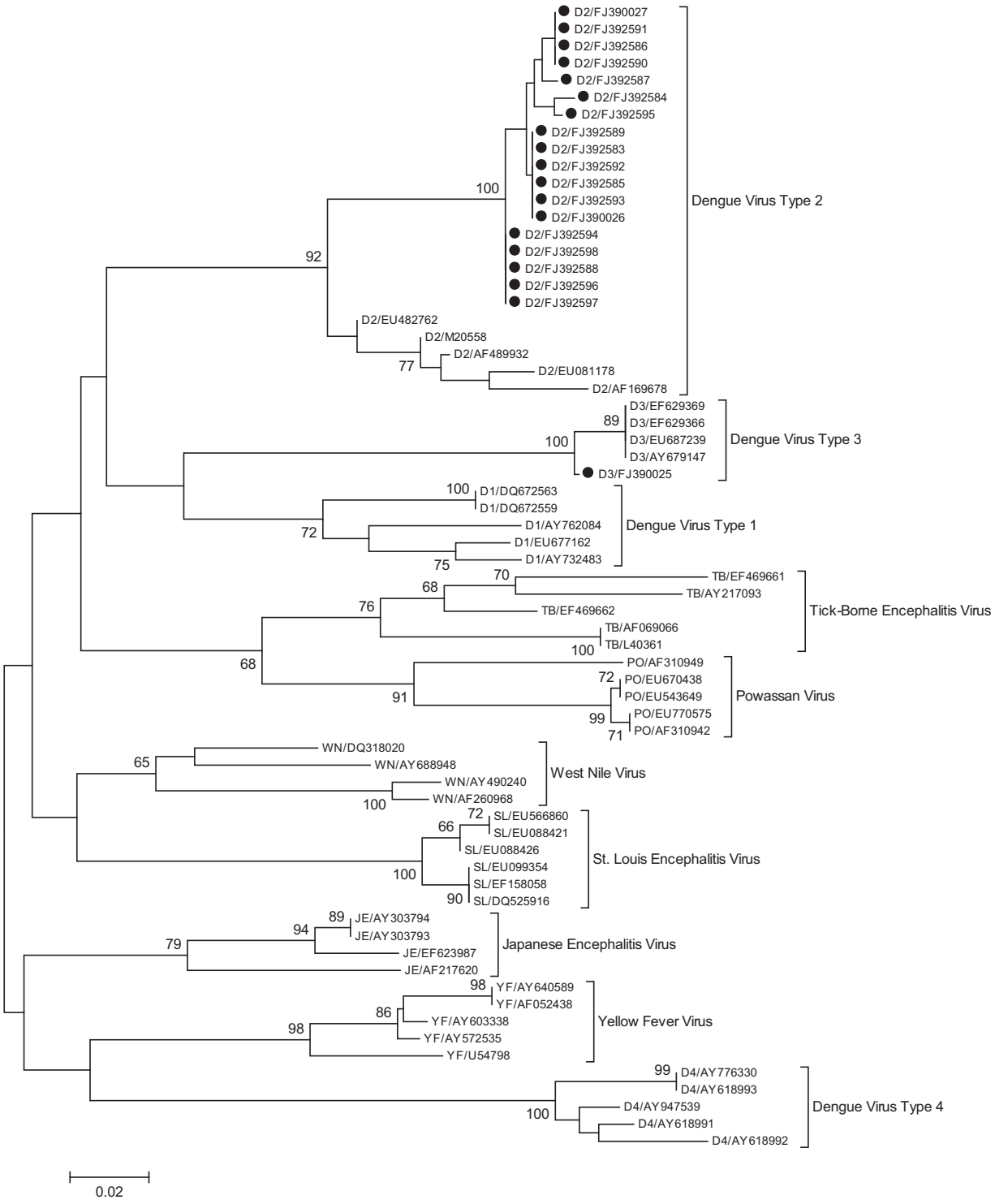

Fig. 3. Phylogeny of 67 flavivirus sequences using 143 nucleotides from the NS5 gene. Strains are denoted by accession numbers from Genbank and viruses are indicated by brackets. Horizontal branch lengths are drawn to scale. Bootstrap values of statistical support for major branches are shown as percentage equivalents.

4. Discussion

The State of Rio de Janeiro is an important epidemiological site for the introduction of flaviviruses, as observed previously for DENV-1 (1986), DENV-2 (1990), and DENV-3 (2000) (Schatzmayr et al., 1986; Miagostovich et al., 1993; Nogueira et al., 2005). Currently, the emergence of new flaviviruses has become an important issue of great public health concern due to the recent incursion and continued transmission of WNV in the Americas (Hayes et al., 2005; Berrocal et al., 2006; Morales et al., 2006; Bosch et al., 2007). In Brazil, the first isolation of SLEV from a human case that was thought to be dengue occurred in the city of São Pedro, State of São Paulo (Rocco et al., 2005). Previous to this case, only two SLEV human infections had been reported in Brazil, both in the Amazon basin (Vasconcelos et al., 1998; Figueiredo, 2000). However, an outbreak of SLEV occurred concomitantly with a large DENV-3 outbreak in São José do Rio Preto, State of São Paulo. During this outbreak, some SLEV patients showed hemorrhagic manifestations

identified by a positive tourniquet test, petechiae, and bleeding (Mondini et al., 2007). Sporadic cases of encephalitis, like those caused by the enzootic flaviviruses, require effective surveillance programs to identify areas of transmission and to enable the immediate implementation of procedures aimed at reducing transmission to humans, including vector control and vaccination.

Several RT-PCRs have been developed for detection of flavivirus RNA by using different pairs of primers for differentiating between species of viruses (Eldadah et al., 1991), including flavi-universal primers for mosquito-borne flaviviruses (Tanaka, 1993) and seven published primers pairs permitting complete detection of the *Flavivirus* genus (Scaramozzino et al., 2001; Chang et al., 1994; Chow et al., 1993; Fulop et al., 1993; Kuno, 1998; Meiyu et al., 1997; Pierre et al., 1994). The generic RT-PCR applied in this study is a very useful method for detection of a wide spectrum of flaviviruses (Sánchez-Seco et al., 2005) and can be used as a complementary method for diagnosing these viruses in Brazil. The amplified flavivirus is identified by sequencing the resulting fragment, and the sequence obtained is compared with those of known flaviviruses in order to identify with precision the detected virus, as shown in Fig. 3. Here, this assay was thoroughly evaluated using a large number of clinical samples during a dengue outbreak in Rio de Janeiro in 2008. The comparison of generic RT-PCR with conventional RT-PCR revealed lower sensitivity of generic RT-PCR, which may be attributed to degenerated primers. As expected, the application of IgM serology showed the importance of serodiagnosis after the eighth day of illness (Fig. 2). These results clearly indicate that different methods should be included in flavivirus surveillance programs, including virological and serological approaches.

Phylogenetic trees of flaviviruses derived from NS5 gene sequences have been described previously (Kuno, 1998; Lanciotti et al., 1999; Scaramozzino et al., 2001; Sánchez-Seco et al., 2005). Furthermore, the phylogenetic tree designed by comparison of the amplification of the nested products in the NS5 location (143 bp) allowed finding all of the complexes of medical interest: the DENV, JEV, YFV, and TBE groups. By using this method, it was possible to identify DENV in 19 patient samples (Fig. 3).

Finally, this generic RT-PCR strategy coupled with DNA sequencing represents a valuable tool for the molecular diagnosis of flaviviruses. However, a definitive identification obviously requires both complete sequencing and the appropriate expertise in flavivirus identification.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

Acknowledgments

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